Optimization of Annealing Temperature for Amplification of 507 bp fragment of *rpoB* Gene of Clinical Multidrug-Resistant *Mycobacterium tuberculosis* Isolate 86

[1]Rusyanthini, E.P., [2]Wirajana, I.N., and [3]Yowani, S.C.

[1] Student Department of Pharmacy, Udayana University, Bali-Indonesia

[2]Department of Chemistry,Udayana University, Bali-Indonesia

[3]Department of Pharmacy, Udayana University, Bali Indonesia

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ABSTRACT

The gene of *rpoB* is the primary gene that is well known as a surrogate marker in MDR-TB (Multidrug-Resistant Tuberculosis) detection. The mutation of *rpoB* was studied around the world in its core region called RRDR (Rifampicin Resistance Determining Region). To learn the mutation in this fragment, PCR (Polymerase Chain Reaction) is the most common method used. This study was purposed to optimize the annealing temperature in amplifying the 507 bp fragment of *rpob* gene containing RRDR of isolate 86.

DNA of MDR-TB isolate 86 was isolated by using Boom method for further amplified by PCR. The oligonucleotide primers used in this study were FrTB and RrTB. Eight different annealing temperature were used to optimize the amplification of *rpoB* gene : 53, 55, 57, 58, 59, 60, 61, and 63^{o}C. Detection of PCR products was done with 1,5% agarose gel electrophoresis.

Agarose gel electrophoresis of PCR products showed that 507 bp of *rpoB* gene could be produced at all annealing temperatures. A faint amplified product was observed at temperature

53 and 55°C. However, at temperature 58 and 60°C more intense bands were observed. In conclusion, the best annealing temperature was at 60°C in producing the 507 bp fragment.

Key words : *rpoB gene*, PCR, *Clinical MDR-tuberculosis isolate*

Correspondence : Rusyanthini, E.P.

Student Department of Pharmacy, Udayana University, Bali-Indonesia

Email : ekarusyanthini@gmail.com

INTRODUCTION

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* and not only infects lung, but also infects other organs. In 2010, approximately 8,8 million of TB cases were reported from around the world. Indonesia was ranked 4th in the world after India, China, and South Africa. It was estimated there were 690.000 cases of TB cases with 640.000 deaths in Indonesia. Control of TB has become complicated by the appearance of multi-drug resistant (MDR) strains of *M. tuberculosis*. In Annual report on global TB control in 2011 by WHO, Indonesia was included in 27 countries that are categorized as high-burden countries for MDR-TB. MDR-TB is caused by *M. tuberculosis* strains resistant against two important and most effective first-line anti-TB drugs, namely rifampin and isoniazid.[1,2]

Rifampin is the first line anti-TB drug which specifically interferes transcription and elongation by binding to the β-subunit of bacterial RNA polymerase.[3] Resistance to rifampin is associated with a various mutation in rifampin resistance-determining region (RRDR) of the *rpob* gene that encode the β-subunit of bacterial RNA polymerase.[4] Studies have shown that 91% of rifampin resistance isolates are also isoniazid-resistant, so that rifampin resistance is a

surrogate marker for the detection of MDR-TB.[5] Many molecular methods have been developed to detect mutation in *rpoB* gene, including polymerase chain reaction (PCR).

PCR is an enzymatic method to amplify a specific nucleotide sequence from template DNA. This method involves three distinct steps regulated by temperature. These steps are pre-denaturation, amplification cycling, and final extension. Each amplification cycling consists of three stages: denaturation at 93-95^{o}C, annealing at a temperature of 37-60^{o}C and primer extension at 72^{o}C temperature. PCR optimization process could be done by varying the conditions used in the PCR process, one of them is annealing temperature. The good result of a PCR depends on the specificity of primer to anneal only to the target sequence, therefore it is important to optimize this molecular interaction. Annealing temperature could be calculated based on the length and composition of nucleotide sequence of primers. Since the annealing temperature does not exactly proper to the template, there will be mispriming and misproduct resulting.[6] In this study, the annealing temperature of primers FrTB and RrTB was optimized in amplifying 507 bp of *rpob* gene containing RRDR from isolate 86.

MATERIALS AND METHOD

Bacterial strains and culture conditions

MDR-TB isolate was collected from Clinical Microbiology Laboratory of Sanglah Hospital, Bali. The isolate was cultured on Lowenstein-Jensen medium.

DNA isolation

DNA was isolated as described by Boom *et al*.[7] Lysis buffer containing of guanidine thiocyanate, Tris-HCl, NaOH, EDTA and Triton-X was used for sample lysis and then added 40 mL diatoms suspension. It was shaken with shaker and centrifuged. Supernatant was discarded.

Pellet was washed twice with 0.5 mL of buffer L2 (GuSCN and Tris-HCl) and once with 1 mL of 70% cold ethanol and 1 mL of acetone. It was dried for 15 minutes at 56^{o}C. RNAse-free water was added into it and incubated at 56°C for 10 minutes for further centrifuged. Supernatant was used as a PCR template. This process was conducted in the Biological safety cabinet.

Amplification of rpoB gene of Mycobacterium tuberculosis

The oligonucleotide primers used in this study were FrTB (5' GTC GAC GCT GAC CGA AGA AGA C 3') and RrTB (5' GAG CCG ATC AGA CCG ATG TTG G 3'). These primers were used to amplify a 507 bp fragment from a conserved region within the *rpoB* gene. The reaction was performed in an automated thermal cycler (Veriti® Thermal Cycler). The PCR programs were performed with an initial denaturation at 95°C for 15 minutes, followed by 40 cycles of denaturation at 94°C for 1 minute, annealing at 53, 55, 57, 58, 59, 60, 61, and 63°C for 1 minute, extension at 72°C for 1 minute; and final extension at 72°C for 10 minutes.

Gel electrophoresis

PCR products were detected on 1,5% agarose gel electrophoresis. Ethidium bromide was added to the gel. The volume 3 µl of PCR product was loaded on the well of the gel and run at 60 volts for 45 minutes. DNA ladder used was 100 bp (Invitrogen™). The band of DNA was visualized under UV light and documented by Bio-Rad Gel Doc™ XR.

RESULTS

Electroforegram of PCR product showed bands at size of target at all varying temperatures (Figure 1 and 2). The fragment was successfully amplified from DNA template. Data from PCR experiments demonstrated that the PCR products of 507 bp were produced at temperature from 53 up

to 60°C. The intensity increases as well as the increasing of annealing temperature.

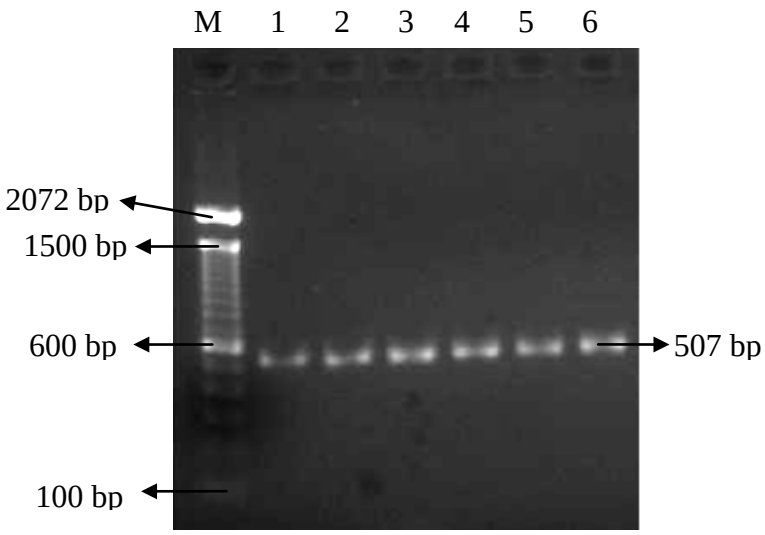

Figure 1
The electroforegram of PCR product at varies annealing temperatures. (1) 53°C, (2) 55°C, (3) 57°C, (4) 59°C, (5) 61°C, (6) 63°C, and (M) DNA ladder 100 bp

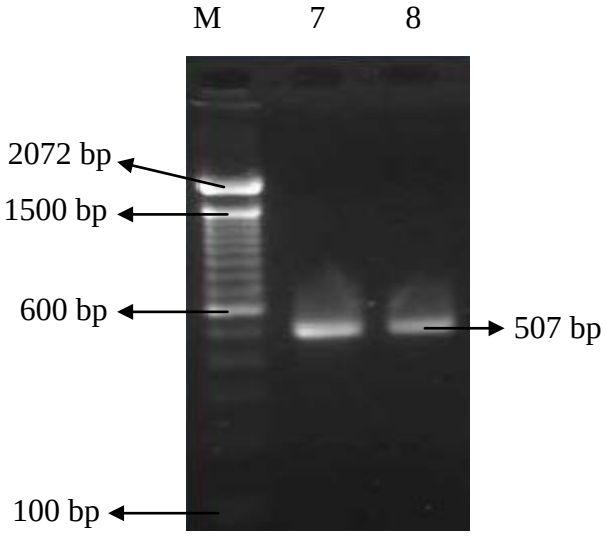

Figure 2
The electroforegram of PCR product at varies annealing temperatures. (7) 58°C; (8) 60°C; and (M) DNA ladder 100 bp

DISCUSSION

PCR is a technique to duplicate the amount of double-stranded DNA targets that involves several repeated stages. It is important to optimize the annealing temperature because the success of a PCR depends on the specificity with which a primer anneals only to its target sequence. If PCR condition is optimal, an intense sharp band of the expected size are should be able to visualize.[8] Effect of annealing temperatures towards the success of PCR process could be seen in the results of the experiment (Figure 1 and 2). Based on data experiment, the bands of DNA on agarose gel electrophoresis only had size 507 bp. This means that the primer used was specific because this primer only amplify the target sequence and not produce unspecific band.

In silico analysis by using program of Clone Manager Suite recommend to use annealing temperature at 60°C. By using this temperature, primer was expected to anneal

more specific and produce more products. Data experiment also proved this temperature was optimum in amplifying the 507 bp of *rpoB* gene because at this temperature an intense amplified product was observed. The faint DNA bands at 53 and 55^{o}C were also observed. It means that temperature was not optimum in amplifying the 507 bp of *rpoB* gene.

Amplification of rpoB gene had also been done on the various isolates in different countries. The amplification of 250 bp fragment of *rpoB* gene containing RRDR of rifampin resistant *M. tuberculosis* isolates in Iran was done by using primer rpob F (5' GGT CGG CAT GTC GCG GAT GG 3') and rpob R (5' GTA GTG CGA CGG GTG CAC GTC 3'). The annealing temperature used was at 62^{o}C.[9] Unlike the amplification of the 250 bp fragment of *rpoB* gene in Iran, the 157 bp region of MDR-TB isolates from Pakistan including RRDR of *rpoB* gene was amplified using annealing temperature at 55^{o}C. Primers sequence used were 5' TGC ACG TCG CGG ACC TCC A 3' (TR8) and 5' TCG CCG CGA TCA AGG AGT 3' (TR9).[10] Different annealing temperature was also used to amplify the 411 bp of *rpoB* gene of rifampin resistant *M. tuberculosis* isolates in China. By using primer rpoB F (5' TAC GGT CGG CGA GCT GAT CC 3') and rpoB R (5' TAC GGC GTT TCG ATG AAC C3'), the annealing temperature was at 58^{o}C.[11] It showed that different annealing temperature was used to amplify the fragment of *rpoB* gene depend on the primers used, therefore optimization of annealing temperature is needed to produce the target fragment.

CONCLUSION

Primer FrTB and RrTB had given the best result at optimal annealing temperature at 60^{o}C, to amplify the 507 bp fragment of *rpoB* gene.

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